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Divalent Cation Binding to Phospholipids: An EPR Study

J.S. Puskin

Department of Radiation Biology and Biophysics
University of Rochester School of Medicine and Dentistry Rochester, New York 14642

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Summary. Divalent cation association to sonicated phospholipid liposomes has been examined with electron paramagnetic spectroscopy. Spectra were obtained suggesting that, in some cases, divalent cations associated with acidic phospholipid head groups are highly mobile.

Using the amplitude of its characteristic sextet signal as a measure of free $Mn(H_2O)_6^{++}$, the apparent affinities of cardiolipin and phosphatidylserine for Mn^{2+} were measured as a function of monovalent electrolyte. Monovalent cations having smaller nonhydrated radii were more effective in displacing Mn from the phospholipids. Under conditions of low divalent cation concentrations, it is shown that the Gouy-Chapman diffuse double layer theory predicts a Mn-affinity (K_A) inversely proportional to the square of monovalent salt concentration. Although this relationship was closely obeyed for Mn binding to cardiolipin, the fall-off in K_A with added sodium chloride was slower in the cases of Mn binding to phosphatidylserine or phosphatidic acid.

When phosphatidylcholine or cholesterol was incorporated into mixed vesicles along with a fixed amount of charged phospholipid, the Mn-binding strength was roughly proportional to the weight fraction of the latter. This result is consistent with: (1) a random dispersal of lipids in the bilayer, and (2) a 1:2 divalent cation-phospholipid interaction.

Cations, especially divalent cations, strongly influence membrane properties such as permeability, adhesiveness, rigidity, sensitivity to electrical stimuli and chemical agents. The cations can act to modify the electrical potential near the surface, to decrease membrane lipid fluidity (Verkleij *et al.* 1974; Jacobson & Papahadjopoulos, 1975), to cause clustering of certain membrane components (Ohnishi & Ito, 1973), and possibly to bridge anionic groups in a single membrane or in two different membranes.

There are two categories of electrostatic interactions between cations and negatively charged surfaces. First, there is the usual type of electrostatic binding where the cations complex to anionic surface moieties. There exists, however, a second type of association, often referred to as

"screening". In this case the cations remain mobile, being held loosely in a "diffuse layer" close to the surface. (Even conceptually, it is not always possible to discriminate completely between complexing and screening, e.g., when discussing transient substitution of surface ligands into the outer hydration shell of a cation. Thus the terms "binding" and "association" will be used sometimes in this paper where the distinction between complexing and screening is unimportant or unclear.)

Screening phenomena were first treated mathematically by Gouy (1910) and Chapman (1913) (*see Appendix*). Although the Gouy-Chapman double layer theory is based on unrealistic assumptions, and more sophisticated formulations are being developed (Stigter, 1975), it remains a convenient semiquantitative framework for discussing the electrostatic interactions between ions and a charged surface. One important application of the theory has been to calculations of surface potentials and conduction-voltage characteristics of excitable membranes (Gilbert & Ehrenstein, 1969). It has also been used to compute the electrokinetic properties of membranes (Cole, 1969; Seaman, 1973).

As discussed in the Appendix, the ionic concentrations in the diffuse layer near a charged membrane may differ greatly from those in the bulk solution. All membrane phenomena dependent on the surface ionic concentrations are affected accordingly. These may include ion transport, enzymatic activity and, significantly, ion-complexation to the surface. Therefore, ion-complexing to membranes cannot be understood without reference to screening. Using the Gouy-Chapman theory in this light, McLaughlin, Szabo & Eisenman (1971) were able to explain the dependence of monovalent cation permeability through black lipid membranes on surface charge and divalent cation concentration. From this analysis they also derived indirect estimates of divalent cation-complexing to several phospholipids.

EPR has been widely employed as a tool for measuring cation binding. The paramagnetic ion Mn^{2+} has proved most useful in this regard because the free aquoion produces an intense sextet signal which is easily monitored (Cohn & Townsend, 1954). Moreover, its spherical symmetry and its ionic radius make Mn^{2+} an excellent analog to Ca^{2+} or Mg^{2+} for probing the role of divalent cations in biological systems. In this study, Mn^{2+}-binding to phospholipids, as modulated by lipid composition and monovalent salts, was measured with EPR.

Because of its sensitivity to the rotational mobility of a paramagnetic ion, EPR can also be used to test whether an ion is complexed to a surface or whether it is in a diffuse double layer. With this technique it

has been found that Cu^{2+} and Mn^{2+} ions associated with certain cation exchangers are highly mobile (Umezawa & Yamabe, 1972; von Goldammer, Müller & Conway, 1973). Spectra obtained from such preparations nevertheless differ noticeably from the respective free Cu^{2+} and Mn^{2+} aquoion spectra at the same temperature. Apparently, the ions loosely associated with the resins are partly restricted in their mobility by weak electrostatic interactions with the fixed anionic groups and perhaps by some partial ordering of surface water molecules. Evidence supporting a similar type of association between divalent cations and some phospholipids will be presented here.

Materials and Methods

Phospholipids isolated from natural sources (assayed as pure by thin layer chromatography) were purchased from Grand Island Biological Company. (The essential features of Mn-binding to phosphatidylserine and cardiolipin shown in Figs. 2 and 3 were confirmed using phospholipids from Nutritional Biochemicals and Sigma, respectively.)

For each sample, a chloroform or hexane solution of the lipid (or lipid mixture) was dried down with a stream of nitrogen. Traces of solvent were removed by pumping in an evacuated chamber for at least 1 hr. The desired aqueous solution was added to each tube; the sample was then sealed, vortexed, and sonicated in a water bath until the suspension clarified. Samples containing only lipids with a net charge usually were clear after a 2–5 min sonication period. A few samples (noted in the text) remained cloudy even after sonicating for 30 min.

EPR spectra of the samples were recorded at $\approx 22\,^{\circ}C$ on a Varian E-12 spectrometer, operating at X-band. Each measurement of signal amplitude or g was determined relative to a pitch standard located in the other chamber of the dual cavity.

Mn-binding to a phospholipid was determined from observed intensities of the characteristic free Mn signals in the presence and absence of vesicles. A large excess of phospholipid was employed in all cases. Competition and cooperativity complications (*see* Hauser, Darke & Phillips, 1976) were thus minimized, so that the affinity could be estimated from the ratio of bound to free Mn:

$$K_A \simeq [\mathrm{Mn}]_{bd}/([\mathrm{Mn}]_{\mathrm{free}}[S])$$

where $[S]$ is the concentration of binding sites. The affinities were, unless stated otherwise, normalized to one lipid phosphorous per site.

A low Mn/phospholipid ratio also afforded these advantages: (a) vesicle precipitation was avoided, (b) accuracy in determining K_A was improved, (c) as discussed in detail in the Appendix, comparisons were more easily made between experimental results and theoretical predictions regarding divalent cation binding to anionic surfaces.

Results and Discussion

Cu^{2+}- and Mn^{2+}-Phospholipid Spectra

Cu^{2+} Spectra. Fig. 1*a* and *b* show spectra from samples containing Cu^{2+} plus phosphatidylserine (PS) and cardiolipin, respectively. The

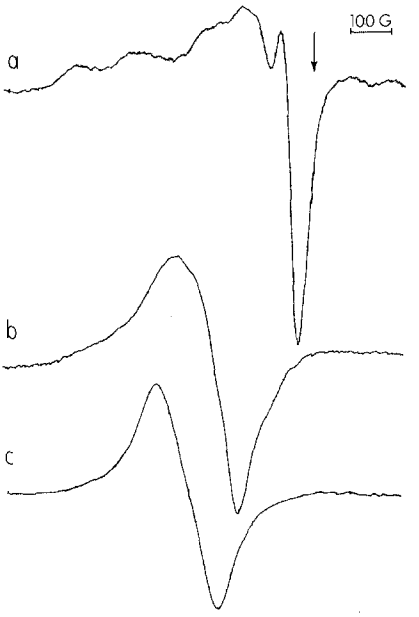

Fig. 1. Spectra of phospholipid-associated Cu^{2+}. Samples contained (in mM): (*a*) 10 PS, 3 HEPES and 0.45 $CuSO_4$; (*b*) 7.6 cardiolipin, 3 HEPES and 0.49 $CuSO_4$; (*c*) 0.9 $CuSO_4$. In (*a*) and (*b*) the pH was adjusted to 7.2 with NaOH. Gains are arbitrary; the arrow indicates a pitch marker

spectra were taken under conditions of low salt and high phospholipid concentrations, which minimize the free Cu^{2+} spectral component (Fig. 1*c*).

With PS a "powder spectrum", indicative of a Cu^{2+}-complex having axial symmetry and little or no rotational mobility, was observed. The splitting parameters, $g_{\|} \sim 2.24$, $g_{\perp} \sim 2.045$, and $A_{\|} \sim 140\,G$, are characteristic of complexes where the bonding, particularly the in-plane π bonding, is mainly ionic rather than covalent (Kivelson & Neiman, 1961; Gersmann & Swalen, 1962).

Cu^{2+} associated to cardiolipin seems to have considerably more rotational freedom than that associated to PS. Fig. 1*b* exhibits those features expected of a spectrum arising from a population of $Cu(H_2O)_6^{++}$ ions, undergoing rapid reorientation of the symmetry axis so as to average the *g*-anisotropy, but having less rotational mobility than the free ion. The (isotropic) $\langle g \rangle$ estimated from Fig. 1*b* is 2.16 ± 0.02, in close agreement with the value $\langle g \rangle = 2.176$ for $Cu(H_2O)_6^{++}$ (Poupko & Luz; 1972) and with the $\langle g \rangle$ in Fig. 1*c*. The peak-to-peak line width $\sim 150\,G$ is also consistent with the total splitting of $\sim 110\,G$ for the $Cu(H_2O)_6^{++}$ hyperfine quartet.

By comparing the line shape in Fig. 1*b* with spectra from Cu^{2+} dissolved in viscous media, the mobility of the cardiolipin-associated Cu^{2+} can be estimated. To aid in this analysis, a series of standards was prepared, composed of dilute copper sulfate in aqueous sucrose solutions. There was a strong similarity between EPR spectra of these standards and Fig. 1*b* when the sucrose concentration was in the range 55–70% by weight. Assuming correspondences between microscopic and macroscopic viscosities for solutions of small molecules (*see*, e.g., Burlamacchi, 1971) and between spectral shape and tumbling rate when comparing the cardiolipin sample to the standards, it follows that the rotational correlation time for cardiolipin associated Cu^{2+} is 50–500 times that for free Cu^{2+}; thus, $\tau_r \sim 10^{-9} - 10^{-8}$ *s* (Morgan & Noble, 1959). Another estimate of τ_r can be derived from published spectra (Kneubühl, 1960) of Cu^{2+} in glycerol, a medium in which the splitting parameters are fairly comparable to those of aqueous copper solutions. Fig. 1*b* is very similar to the spectrum of Cu^{2+} in glycerol at 325 °K, where $\eta \sim 130$ cP. Assuming an ionic radius of ~4.5 Å for the Cu^{2+}-glycerol complex, $\tau_r \sim 3 \times 10^{-9}$ *s* is calculated from the Debye relationship.

In summary, the spectra in Fig. 1 suggest that Cu^{2+} is immobilized by binding to PS, but is loosely held by cardiolipin in the form $Cu(H_2O)_6^{++}$. The binding of Cu^{2+} to cardiolipin is less simple than the conventional view of a diffuse layer phenomenon. Tumbling of Cu^{2+} ions is noticeably slowed by cardiolipin, perhaps through the formation of a short-lived outer sphere complex. Cu^{2+} mobility may also be affected by steric hindrance or ordered water, e.g., in the spaces intercalating between phospholipid head groups. It should be noted also that the terms screening and complexation refer to an instantaneous view of cation binding, while EPR necessarily entails an averaging over the spin-relaxation time T_2.

Mn^{2+} Spectra. EPR spectra from samples containing Mn^{2+} plus PS and cardiolipin, respectively, are shown in Fig. 2*a* and *b*. There is no visible spectral contribution from the narrow (~25-G wide) hyperfine sextet lines characteristic of free Mn in water at ~22 °C (Cohn & Townsend, 1954). That essentially all the Mn^{2+} is associated with the vesicles was confirmed by obtaining spectra indistinguishable from those in Fig. 2*a* and *b* when the respective phospholipid concentrations were increased several-fold, other conditions being held constant. This result furthermore implies that the spectra are not affected by spin-spin interactions between Mn^{2+} ions.

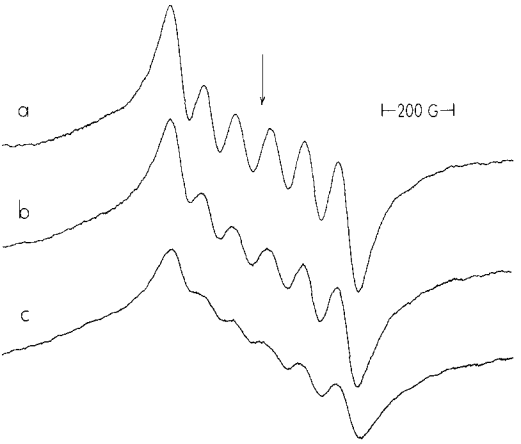

Fig. 2. Spectra of phospholipid-associated Mn^{2+}. Experimental conditions (in mM): (*a*) 4.1 cardiolipin, 0.26 $MnCl_2$; (*b*) 7.25 PS, 0.32 $MnCl_2$; (*c*) as in (*b*), except with 0.3 ml glycerol/ml. Samples also contained 10 mM Na-HEPES at pH $\simeq$ 7.3. The pitch line is indicated by an arrow

Neither Fig. 2*a* nor *b* shows evidence of "powder spectrum" features (forbidden transitions or static zero field splittings (zfs)) characteristic of most Mn^{2+}-complexes having low rotational mobility (Reed & Cohn, 1970; Reed & Ray, 1971). Instead each spectrum can be simulated by a set of six identical lorentzian lines separated by the same hyperfine splittings as the free Mn sextet. The computer generated spectra which most accurately matched the experimental spectra had line widths of $\simeq$ 90 G for cardiolipin and $\simeq$ 110 G for PS. (These values refer to a peak-to-peak width of an individual line, unaffected by overlap.) The evidence cited here suggests that spin-relaxation rather than static zfs is the main determinant of line shape in both Fig. 2*a* and *b*.

When glycerol was added to either the PS or the cardiolipin suspension, the absorption lines were "smeared out" further (shown for PS in Fig. 2*c*). This increase in line-width with increased viscosity strengthens the conclusion reached above, and indicates that the Mn^{2+} spin-relaxation mechanism is influenced by fluctuations having a correlation time shorter than the reciprocal of the Larmor frequency; i.e., $\tau_c < 1.7 \times 10^{-11}$ *s* (Burlamacchi, 1971; Reed, Leigh & Pearson, 1971).

The line shapes and their dependence on viscosity strongly suggest that the Mn^{2+} ions associated with PS and cardiolipin are in a rotationally mobile form (Reed *et al.*, 1971). Thus the Mn^{2+}-association to these lipids is probably similar in nature to that described for Cu^{2+}-binding to cardiolipin or Mn^{2+}- and Cu^{2+}-binding to some ion-exchangers. Such

an interpretation is less certain in this case, however. It is theoretically possible that Mn^{2+} is strongly immobilized by the phospholipids, but that the static zfs are so small as to be obscured by the relaxation broadening. Such a complex must involve very little static distortion of the Mn^{2+} coordination shell from octahedral symmetry, but would nevertheless have to undergo collisions with solvent molecules, producing sizable fluctuations in zfs with a frequency, $1/\tau_c > 10^{11}\ s^{-1}$.

Dependence of Mn^{2+}-Binding on Monovalent Cations

Because of its narrowness and its relatively large amplitude, the EPR signal due to free Mn^{2+} can be readily detected and quantitated, even in the presence of a much larger fraction of bound Mn^{2+} ions (Cohn & Townshend, 1954). In this way the displacement of Mn^{2+} from phospholipid vesicles was monitored, and the binding affinity deduced (*cf. Materials and Methods* and *Appendix*) over a range of conditions. In those cases where the broader bound signal introduced appreciable distortion of the free signal, its contribution was subtracted out using the appropriate spectral shape (Fig. 2*a* and *b*). This correction never exceeded 25%, and was less than 15% except for samples in which the monovalent salt concentration was <0.1 M or in which choline or tetramethylammonium (TMA^+) was substituted.

Influence of Monovalent Cation Radius. Table 1 compares a number of monovalent cations in their abilities to displace Mn^{2+} from phospholipid vesicles (displacement of Mn by the small amount of Na in the HEPES buffer was shown to be negligible). As would be expected from previous results (Abramson *et al.*, 1966), the sequence: $Li^+ > Na^+ > TMA^+$ was found. In general, the smaller the nonhydrated radius of monovalent cation, the stronger its inhibition of Mn^{2+}-binding. Na^+, K^+, Rb^+ and Cs^+, however, were equally effective in displacing Mn^{2+} from cardiolipin and they differed only slightly in displacing Mn^{2+} from PS. The trend observed with decreasing radius, it should be noted, does not necessarily imply any disruption of the monovalent cations' inner hydration shells during interaction with the phospholipids.

Effect of Varying $[Na^+]$. Plotted against $[Na^+]$ in Fig. 3 are affinities of PS and cardiolipin for Mn^{2+}, derived from EPR measurements of free $[Mn^{2+}]$. Assuming no Na-phospholipid complexing, the Gouy-Chapman theory predicts that for the range of conditions employed in

Table 1. Inhibition of Mn^{2+}-binding by monovalent cations

Monovalent salt	Mn-cardiolipin		Mn-PS	
$M^+(Cl^-)$	K_A^{-1}(mM)	Relative K_A^{-1}	K_A^{-1}(mM)	*Relative* K_A^{-1}
Li^+	1.39[a]	2.36	0.510[c]	3.25
Na^+	0.59[a]	1.00	0.157[c]	1.00
K^+	0.60[a]	1.02	0.140[c]	0.89
Rb^+	0.61[a]	1.03	0.126[c]	0.80
Cs^+	0.59[a]	1.01	0.113[c]	0.72
TMA^+	0.082[b]	0.14	0.022[d]	0.14
$Choline^+$	0.111[b]	0.19	0.021[d]	0.13

The measurements and calculations of K_A were done as described in *Materials and Methods*. The concentration of monovalent salt was 120 and 145 mM in the cardiolipin and PS cases, respectively.

[a] The cardiolipin concentration was 2.63 mM.

[b] Cardiolipin concentration was 0.66 mM.

[c] The [PS] was 3 mM.

[d] The [PS] was 1.5 mM.

(The vesicle concentrations were reduced when monitoring Mn-displacement by organic cations to avoid interference from bound Mn signals.) The sample also contained 16 mM Na-HEPES (pH 7.3) plus enough manganese chloride to make the molar ratio of Mn to phospholipid phosphorous 1:32.

Fig. 3, a graph of measured $\log K_A$ *vs.* $\log [Na]$ should be a straight line with slope $s = -2$. This follows from Eq. (9) and (11) of the *Appendix*, based on a surface charge density σ of 1 net charge per $\simeq 50$–$60\,Å^2$, given any mixture of Mn^{2+}-screening or-complexing. The theoretical line will be shifted up or down, depending on σ and on K_∞, the Mn-phospholipid affinity [*see* Appendix: Eq. (11)] independent of surface potential effects which concentrate Mn^{2+} near the binding sites. For comparison it should be noted that with a simple (1:1) competition between Mn^{2+} and Na^+ for isolated identical binding sites, one expects $-1 \leqq s \leqq 0$.

The observed [Na] dependence of Mn-binding to cardiolipin (Fig. 3) is in excellent agreement with the Gouy-Chapman theory. Plots from 6 independent experiments on 3 different batches of cardiolipin each showed a linear dependence of $\log K_A$ on $\log [Na]$ in the region around 150 mM NaCl. The estimated slopes were, furthermore, all in the range $s = -1.99 \pm 0.08$, a variation accounted for by uncertainties in EPR quantitation. Also of significance is the finding that the measured K_A's were <3 times larger than affinities K_{DL} (dashed curve) calculated from Eq. (9), assuming divalent cation screening only. This supports the sug-

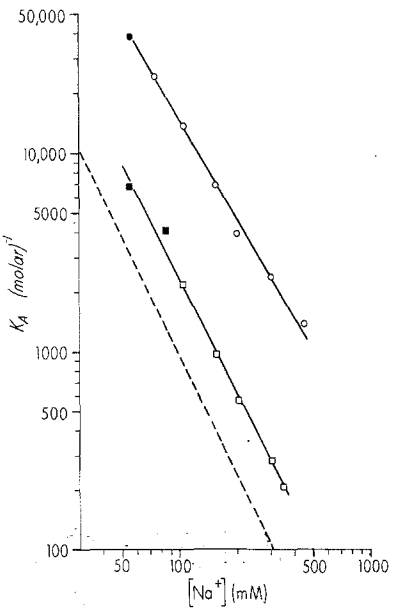

Fig. 3. Mn-binding to cardiolipin and PS as a function of medium [Na]. K_A determinations were as described in *Materials and Methods*. Samples contained: (□) 3.16 mM cardiolipin (6.32 mM anionic sites), 200 μM $MnCl_2$; (○) 3.17 mM PS, 85 μM $MnCl_2$; 16 mM Na-HEPES (pH 7.3) plus varying NaCl. To minimize interference from bound Mn signals at low [Na], (■) and (●) were carried out at 1.5 mM lipid-P and 45 μM Mn, analogous to (□) and (○), respectively. The plotted concentrations represent estimates of [Na] in the bulk medium, as required for testing the Gouy-Chapman Theory (*cf. Appendix*). Subtracting the expected diffuse layer fraction, the medium [Na] was taken to be the total [Na] minus the concentration of phospholipid (net) charge in the sample. The dashed curve is the theoretical "diffuse layer association constant" K_{DL} calculated from Eq. (9), assuming 50 Å^2/ fixed surface charge. (As discussed in the *Appendix*, K_{DL} is the apparent affinity derived by equating the bound fraction with those divalent cations in the diffuse layer.)

gestion made earlier, based on spectral shape and its viscosity dependence, that cardiolipin-associated Mn is fairly mobile.

In contrast, while the PS data also consistently indicated a linear relationship between $\log K_A$ and $\log$ [Na] the experimental slopes deviated significantly (*see* Fig. 3) from $s = -2$. Eight independent determinations of s produced values in the range $s = -1.49 \pm 0.12$. Some variation ($\pm 30\%$) was also noted among the intercepts obtained at 150 mM NaCl. The causes of these variations are unknown, but one factor could be differing fatty acid composition between batches of PS.

The inaccuracy inherent in any one of the Gouy-Chapman postulates listed in the Appendix might be cited as possible explanation for the discrepancy between theoretically and experimentally derived slopes for

Table 2. Mn-binding to PC, PE and PA

Lipid type	NaCl (mM)	K_A(molar)$^{-1}$
PC	150	9.1 ± 4
PE	150	150 ± 30
PA	190	$(5.8 \pm 0.6) \times 10^4$
PA	290	$(3.5 \pm 0.5) \times 10^4$

Each entry for K_A represents the range of values from 3 or 4 measurements. (Much of the imprecision in the Mn-PC determination stems from technical difficulties relating to the very low affinity.) Lipid concentrations varied considerably, but the ratio Mn/phospholipid was 0.02 to 0.04 in each case. The samples were buffered at pH 7.3 ± 0.1 with 16 mM HEPES. (The PA suspensions in 290 mM NaCl remained slightly cloudy after sonication.)

Mn—PS binding. The agreement between theory and experiment found in the case of cardiolipin, however, suggests an explanation based on: (a) some specific Na—PS binding, or (b) complications arising from penetration of cations into the restricted spaces between head groups of PS.

Binding to Other Phospholipids

Summarized in Table 2 are affinities of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidic acid (PA) for Mn, as measured by the EPR method previously applied to PS and cardiolipin. While there was detectable binding to the zwitterionic phospholipids, PC and PE, it was much weaker than to any of the acidic species. No significant changes were found in the PC and PE affinities, moreover, when [NaCl] was lowered to 10 mM. It is likely that these differences mainly reflect the tendency for cations to become highly concentrated near the anionic vesicle surfaces. The binding to PA, on the other hand, was considerably stronger than to either PS or cardiolipin (*cf.* Fig. 3). The higher affinity may reflect some double ionization of PA phosphate groups or may be due to steric factors.

Binding to Mixed Vesicles

Fig. 4 shows the dependence of Mn-binding to acidic phospholipids on added PC in mixed vesicle preparations. It should be noted that the

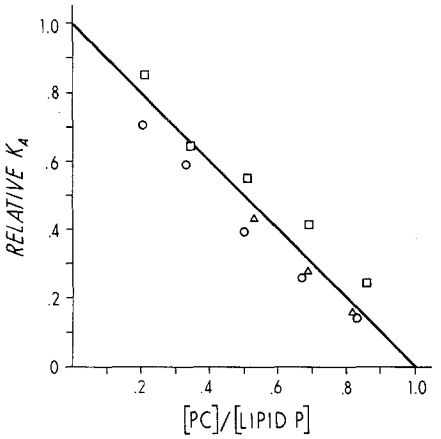

Fig. 4. Mn-binding to acidic phospholipids in mixed vesicles with PC. In addition to 16 mM HEPES (pH 7.3) and varying amounts of PC, the samples contained: (○) 3 mM PS, 0.145 M NaCl, 93 μM Mn; (□) 2.63 mM cardiolipin, 0.12 M NaCl, 163 μM Mn; (△) 0.29 mM PA, 0.19 M NaCl, 12.5 μM Mn. The affinities are normalized to the respective value for each charged phospholipid at zero PC. Small corrections (< 10%) were made for Mn-PC binding, based on the mean affinity from the data in Table 2 ($K_A \simeq 8\,M^{-1}$)

respective charged lipid concentration was held constant in each set of data. Interestingly, a similar behavior was found for each of the lipids tested: to a first approximation (Fig. 4, solid line) an n-fold dilution of the vesicles with PC produced an n-fold reduction in K_A. (In performing these experiments, the two lipids had to be properly mixed before sonication; otherwise, the binding was nearly independent of added PC.)

The roughly linear relationship between K_A and [PC] suggests a model in which: (1) the binding of Mn is strongly enhanced at sites with two adjacent anionic head groups, and (2) there exists a random (or ordered) dispersal of PC throughout the bilayer with little tendency for clustering of one type of molecule. Since one charged head group might only assist in attracting Mn to the surface where it can bind to another, this model does not necessarily imply formation of a 1:2 Mn-phospholipid complex, as proposed by Hauser, Chapman & Dawson (1969). A direct two-point attachment, on the other hand, is not inconsistent with a highly mobile state for PS- or cardiolipin-associated Mn, suggested here, so long as the complex is very short-lived.

As illustrated in Fig. 5, the incorporation of cholesterol into PS vesicles had an effect on Mn-binding similar to that of PC. About twice as much cholesterol as PC was needed, on a molar basis, to produce the same reduction in apparent K_A, however. This means that in both types

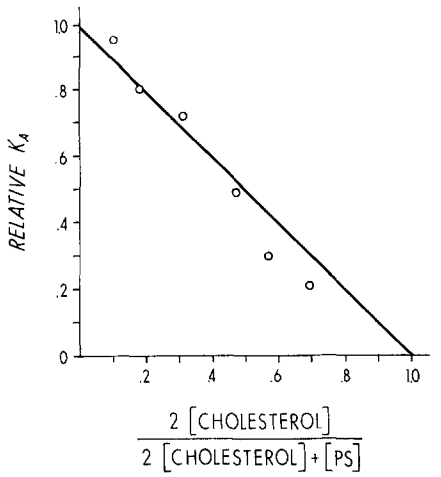

Fig. 5. Mn-binding to PS/cholesterol vesicles. Experimental conditions: 3.8 mM PS, 0.15 M NaCl, 125 μM $MnCl_2$, 10 mM HEPES (pH 7.35) and varying cholesterol. The affinities are presented relative to K_A at zero cholesterol. (The sample containing the largest amount of cholesterol remained slightly cloudy after sonication.)

of mixed vesicles Mn-affinity was roughly proportional to the average surface charge density. The results in Fig. 5 perhaps reflect a lipid packing arrangement in which a pair of cholesterols can displace a single phospholipid molecule from its place in the lattice.

The decreased binding with added PC or cholesterol might be explained in terms of a smaller surface potential without postulating any Mn interaction with pairs of acidic head groups. While a more rapid fall-off in divalent cation binding with decreasing surface charge density is expected, on the basis of the Gouy-Chapman Theory, than is found in Fig. 4 or 5 [*Appendix:* Eqs. (9) & (11)], that prediction is predicated on a uniform, smeared-out charge density. This assumption, a drastic simplification for membranes composed of one particular type of phospholipid, becomes even less realistic when applied to mixed vesicles; therefore, it is not surprising that the theory fails to account for the characteristics of ion-binding to vesicles containing both charged and uncharged lipids.

Discussion

From monolayer studies Hauser *et al.* (1976) estimated the Ca^{2+}—PS affinity to be $\simeq 10^6\ M^{-1}$ at 10 mM NaCl, in the limit of low $[Ca^{2+}]$. This agrees very well with the affinity obtained from extrapolation of the

Mn^{2+}—PS curve in Fig. 3 to 10 mM $[Na^+]$. The same authors also noted a rapid decrease in apparent K_A with increased $[Ca^{2+}]$, presumably due to suppression of the surface potential by Ca^{2+}. Such effects of divalent cations (and H^+) on surface potential may explain why the K_A's measured by some investigators (Hendrickson & Fullington, 1965; Abramson *et al.*, 1966; Haynes, 1974) may be lower than those obtained at comparable monovalent electrolyte concentrations but low M^{2+}/phospholipid ratios and neutral pH.

In comparison to data presented here on binding to mixed vesicle preparations (Fig. 4), Haynes (1974) has reported a very slow fall-off in Ca^{2+}-binding to PA/PC vesicles with added PC. Again the larger divalent cation concentrations employed in that study may account for this apparent discrepancy, since Ca^{2+} is known to induce clustering of charged phospholipids in mixed vesicles (Ohnishi & Ito, 1973). Clustering may have been enhanced, furthermore, by the use of synthetic phospholipids with fully saturated fatty-acids.

In conclusion, EPR provides a direct, rapid method for monitoring the amount of Mn-binding to phospholipid vesicles. The value of this technique is increased by the similarity found between Mn-binding and Ca- or Mg-binding to phospholipids (Haynes, 1974; Hauser *et al.*, 1976). EPR can also be employed, under some conditions, to probe the mobility of surface-associated paramagnetic ions. Both types of EPR observations should be generally applicable to the problem of divalent cation-membrane association. Paramagnetic ion mobility might also provide a good criterion for identifying different classes of binding sites in complex membranes.

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Appendix

Summary of Gouy-Chapman Diffuse Double Layer Theory

Consider a membrane containing fixed negative charges in contact with a uni-univalent salt solution. The electric field set up in the solution near the membrane will induce an increased cation concentration and a

decreased anion concentration in this region (the diffuse layer) compared to the respective ion concentrations $C^{\pm}(\infty)$ in the bulk medium where the field is effectively screened. The spatial distributions of the ions and of the electrical potential can be calculated under a set of simplifying assumptions (Gouy, 1910; Chapman, 1913; Barlow, 1970; Sheehan, 1970).

(1) The fixed charge is spread out uniformly on the membrane.

(2) The electrolyte consists of points ions. Chemical activity corrections due to interactions between the ions are neglected.

(3) The dielectric constant of the diffuse layer is identical to that of the bulk medium.

(4) Complexing of ions (adsorption) to the surface is neglected.

From Poisson's equation, the potential at the surface ψ_o is given by (Sheehan, 1970):

$$\sinh e\psi_o/2kT = Le\sigma/2\varepsilon kT \quad \text{(MKS units)} \tag{1a}$$

where e is the magnitude of the electronic charge, σ is the surface charge density, ε is the dielectric constant and kT has its usual meaning. The Debye length, L, is defined by $L^2 = \varepsilon kT/2e^2 C^{\pm}(\infty)$. Expressing L in Å and σ in electronic charges/Å^2, the equation reduces to:

$$\sinh e\psi_o/2kT \simeq 43 L\sigma \quad \text{at } T \simeq 21\,^{\circ}\text{C}. \tag{1b}$$

The potential $\psi(x)$ at a distance x from the surface is calculated from (Barlow, 1970; Sheehan, 1970):

$$\tanh e\psi(x)/4kT = e^{-x/L} \tanh e\psi_o/4kT. \tag{2}$$

The ionic concentrations at x are:

$$C^{\pm}(x) = C^{\pm}(\infty) \exp(\mp e\psi(x)/kT). \tag{3}$$

The Distribution of a Divalent Cation M^{++} in the Diffuse Layer

The Gouy-Chapman theory can be generalized to accomodate the inclusion of divalent cations in the bathing medium. If the sample divalent cation concentration is much less than $C^{\pm}(\infty)$ and is, moreover, much less than the concentration $[S]$ of anionic binding sites (moles of surface charge/liter of sample), the modification is trivial. The ionic strength and the Debye length will be only slightly altered. Even

allowing for some complexation of M^{++} to the surface, σ will not change appreciably. Thus, since $\psi(x)$ depends only on σ and L, it will not be affected much by these trace amounts of M^{++}. The local concentration of M^{++} will then by given by:

$$C^{++}(x) \simeq C^{++}(\infty) \exp(-2e\psi(x)/kT) \tag{4}$$

where $\psi(x)$ is calculated from Eqs. (1*b*) and (2), ignoring the divalent cations.

Under these conditions (trace quantities of M^{++}), the M^{++}-association with the surface can be calculated in terms of $C^{\pm}(\infty)$ and σ. Although it will be assumed that the monovalent ions do not adsorb to the surface, both M^{++}-screening and -complexing will be considered.

Consider first the divalent cations in the diffuse layer. The number of divalent cations held in the layer by the electric field is given by an integral over the diffuse layer volume:

$$N_{DL}^{++} = \int [C^{++}(x) - C^{++}(\infty)]\, dV_{DL}. \tag{5a}$$

Since $C^{++}(x) = C^{++}(\infty)$ outside the diffuse layer, we can express C_{DL}^{++}, the excess moles of M^{++} in the diffuse layer per liter of sample, as:

$$C_{DL}^{++} = 10^{-27} A \int_0^{\infty} [C^{++}(x) - C^{++}(\infty)]\, dx \tag{5b}$$

where A is the surface area/liter of sample, i.e.,

$$A = \frac{\text{charged sites/liter of sample}}{\text{charged sites/Å}^2 \text{ of surface}} = \frac{6 \times 10^{23}[S]}{\sigma}. \tag{6}$$

Combining Eqs. (4)–(6),

$$\frac{C_{DL}^{++}}{C^{++}(\infty)[S]} = \frac{6 \times 10^{-4}}{\sigma} \int_0^{\infty} [e^{-2e\psi(x)/kT} - 1]\, dx. \tag{7}$$

Since the fixed charge sites are unsaturated with respect to M^{++}, the expression on the left can be interpreted as an association constant. To emphasize that the association of M^{++} with the surface is of the diffuse layer type, the constant will be denoted as K_{DL}.

From Eq. (2) and the definition of the hyperbolic tangent,

$$K_{DL} = 6 \times 10^{-4} \int_0^{\infty} \left[\left(\frac{1 + \ell e^{-x/L}}{1 - \ell e^{-x/L}} \right)^4 - 1 \right] dx \tag{8}$$

where $\ell = |\tanh(e\psi_0/4kT)|$.

Using the substitution $\mathscr{U}=1-\ell e^{-x/L}$, the integral can be solved to yield:

$$K_{DL}=\frac{1.6\times 10^{-3} L\ell(3-3\ell+\ell^2)}{(1-\ell)^3\sigma}. \tag{9}$$

For sufficiently high surface charge densities and low ionic strengths, $\exp(-e\psi_0/4kT)\gg\exp(e\psi_0/4kT)$. Then $\ell\to 1$ and $1-\ell\to 2\exp(e\psi_0/2kT)$. Thus, $K_{DL}\propto L\sigma^{-1}\exp(-3e\psi_0/2kT)$. However, in the same limit, Eq. (1*b*) implies that $\exp(-3e\psi_0/2kT)\propto L^3\sigma^3$. Therefore, for high surface potential $|\psi_0|$, the association constant varies as:

$$K_{DL}\propto L^4\sigma^2\propto\sigma^2/C^{\pm}(\infty)^2. \tag{10}$$

Adsorption of M^{++} to the Surface

The adsorption of M^{++} to the surface is proportional to its concentration near $x=0$. Therefore, the association constant for complexation is given by:

$$K_C=K_\infty e^{-2e\psi_0/kT}, \tag{11}$$

where K_∞ is the binding constant in the absence of diffuse layer effects; i.e., at high ionic strength or low surface charge density. The affinity of the surface for divalent cations can then be defined as $K_A=K_{DL}+K_C$.

For large $|\psi_0|$, it follows from Eq. (1*b*) and Eq. (11) that

$$K_C\propto L^4\sigma^4\propto\sigma^4/C^{\pm}(\infty)^2. \tag{12}$$

Hence, for the conditions cited, whatever the relative strengths of K_{DL} and K_C, the binding affinity for M^{++} varies inversely as the square of the monovalent cation concentration.

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